

Living inside termites: an overview of symbiotic interactions, with emphasis on flagellate protists

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To degrade lignocellulose efficiently, lower termites rely on their digestive tract's specific features (i.e., physiological properties and enzymes) and on the network of symbiotic fauna harboured in their hindgut. This complex ecosystem, has different levels of symbiosis, and is a result of diverse co-evolutionary events and the singular social behaviour of termites. The partnership between termites and flagellate protists, together with prokaryotes, has been very successful because of their co-adaptative ability and efficacy in resolving the needs of the involved organisms: this tripartite symbiosis may have reached a physiologically stable, though dynamic, evolutionary equilibrium. The diversity of flagellate protists fauna associated with lower termites could be explained by a division of labour to accomplish the intricate process of lignocellulose digestion, and the ability to disrupt this function has potential use for termite control. Multi-level symbiosis strategy processes, or the cellulolytic capacity of flagellate protists, may lead to innovative pathways for other research areas with potential spin-offs for industrial and commercial use.

Key words: flagellate protists, hindgut symbiotic fauna, lignocellulose digestion, subterranean termites

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INTRODUCTION

Termites are social insects closely related to cockroaches, from which they evolved (Lo et al. 2000; Inward et al. 2007a; Engel et al. 2009) and are denominated as Polyneoptera a monophyletic group including the cockroaches, Dermaptera, Plecoptera, Orthoptera, Embioptera, Phasmatodea, Mantophasmatodea, Grylloblattodea, Mantodea, and Zoraptera. More than 3,000 species of termites have been described globally, but their areas of high diversity are located in the tropics,

particularly in Africa, South America and Asia (Krishna et al. 2013). Termites can be informally divided into two groups: lower (all families but Termitidae) and higher termites (Termitidae), based on the presence or absence of flagellate protists in their hindgut, respectively, and also on different feeding and nesting habits, and different intestinal compartmentalisation (Eggleton 2011; Hongoh 2011; Krishna et al. 2013). These insects are abundant in many terrestrial ecosystems, particularly in the tropics where they are a dominant invertebrate group that heavily

contributes to the lignocellulose decomposition process – thus have been called ‘ecosystem engineers’ (Eggleton 2011; Palin et al. 2011). Termites also have a major role in diverse ecosystem functions, such as nutrients and organic matter cycling and redistribution, soil fertility promotion, generation and regulation of soil biodiversity and ecosystem restoration (Zimmermann et al. 1982; Bignell & Eggleton 2000; Sugimoto et al. 2000; Jouquet et al. 2011). They are major contributors to the ecologic stability of their habitats. By preparing different substrates, like wood or leaf-litter, into forms easily accessed by microorganisms, termites play a major role as ecosystem conditioners (Lawton et al. 1996). Termites are able to degrade lignocellulose efficiently (e.g. Ohkuma 2008; Husseneder 2010; Watanabe & Tokuda 2010) and their feeding habits span a gradient from sound wood to other lignocellulosic plant materials with different humification gradients, such as plant litter or soil (Sleaford et al. 1996). This niche differentiation has allowed termites to promote an impact on the global terrestrial carbon cycle, exceeding the cumulative decomposition roles of other arthropods (Bignell et al. 1997).

Efficient lignocellulose utilisation as a food source by termites is possible because of the establishment of endo- and ectosymbiosis, including microorganisms of all major taxa. These symbionts are Archaea and Bacteria, and protists: unicellular eukaryotes belonging to two separate lineages, the parabasalids and the oxymonads (Fig.1) (Bignell 2000; Bignell & Eggleton 2000; Brune & Ohkuma 2011; Adl et al. 2012; Brune 2013). Ectosymbiosis has evolved in the fungus-growing termites (Macrotermitinae) which cultivate a basidiomycete fungus (*Termitomyces* spp.) (e.g. Nobre & Aanen 2012), whereas the majority of the other termites rely solely on endosymbiosis. The fauna harboured inside the hindgut assists the termite host with energy metabolism, nitrogen and vitamin supply and also additional defence mechanisms (Salem et al. 2014; Peterson et al. 2015; Zheng et al. 2015).

Higher termites, account for nearly 75% of Isoptera species richness and yet belong to a single family, Termitidae. These termites have retained their bacterial symbionts, but lack the protozoan gut symbionts. They have various feeding habits,

with clear separation of feeding and nesting sites, and exhibit a highly compartmentalised intestine (except for Macrotermitinae and Sphaerotermitinae). Dissimilarly, lower termites feed strictly on lignocellulose and are aided by hindgut symbionts during the digestion process; they are considered to be an ancestral branch of termites which comprises 11 families (Krishna et al. 2013). Lower termites have a dilated section of the anterior hindgut (the paunch) where the bulk of symbiotic microbiota is harboured. Most lower termites nest and feed within the same wood resource. With potential impact on within-nest endosymbiont transmission, lower termites rely on regurgitation of crop contents and saliva (stomodaeal trophallaxis) as well as proctodeal trophallaxis, involving anus-to-mouth exchanges of hindgut fluids, to pass food and gut contents to nest mates, whereas higher termites rely mainly on stomodaeal trophallaxis (Eggleton 2011; Shimada et al. 2013; Mirabito & Rosengaus 2016). Proctodeal trophallaxis fosters the social, nutritional and symbiotic fauna interactions among lower termites belonging to the same colony, probably playing a key role in the integration of the information of these different environments (Nalepa 2015). Trophallaxis may be horizontal, among nestmates, or vertical, among parents and offspring.

LOWER TERMITES GLOBAL IMPACT

Because of their feeding habits and preferences, lower termites have an important ecological impact on diverse ecosystems, but are also considered to be structural, agricultural and forestry pests, as they attack cultivated plants and forest nurseries (Rouland-Lefèvre 2011). Lower termites account for 80% of the economically important species known to cause major problems in artificial constructions (Nobre & Nunes 2007; Rust & Su 2012). There is concern that the number of invasive termite species has increased more than 50% since 1969 (Evans et al. 2013), which may be related to the globalisation of trade. In 2010, the global economic impact of termites was estimated at 35.6 billion euros, and subterranean termites accounted for 80% of this figure, i.e. approximately 24 billion euros (Rust & Su 2012).

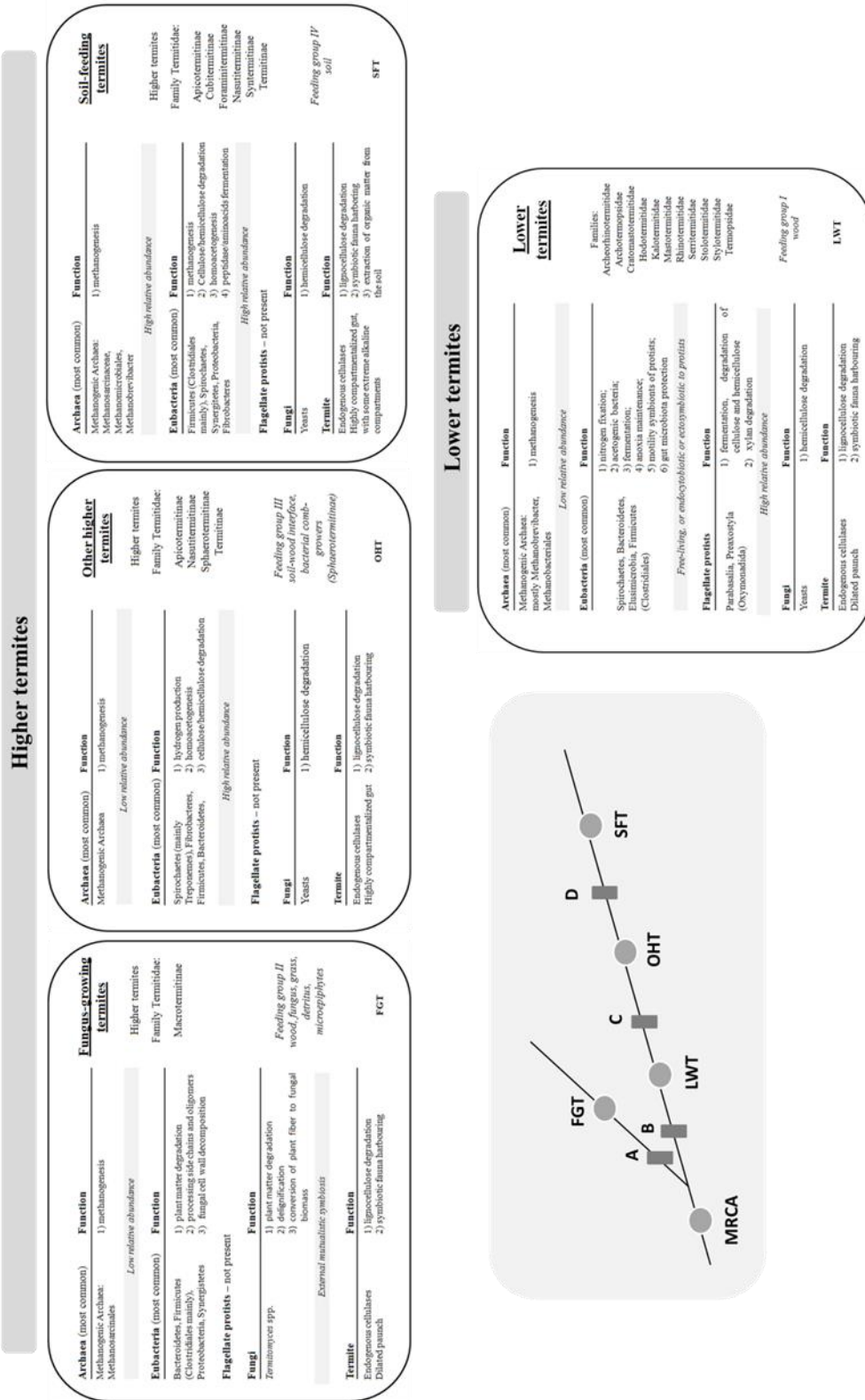


Fig. 1. Possible evolutionary trajectory, from a most recent common ancestor of the cockroach type, of the simplified feeding groups of termites (LWT – Lower termites; FGT – Fungus-growing termites; OHT – Other higher termites; SFT – Soil-feeding termites) based on their symbiotic fauna [based on: Donovan et al. 2001, Eggleton & Taylor 2001, Inward et al. 2007b, Bujang et al. 2014; Otani et al. 2014]. A= acquisition of external symbiosis with basidiomycete fungi; B=acquisition of flagellate protists; C=loss of flagellate protists; D=acquisition of strict soil-feeding habits. Some subfamilies have representatives in more than one feeding group.

As the human population increases, production, trade and use of wooden structures and bio-products susceptible to termite infestation increases, potentially increasing the spread of termite pest species. Warmer seasons and changes in precipitation patterns due to climate change are expected to influence termite territory size and distribution. For example, the known 27 species of invasive termite species are likely to increase their ranges (Su & Scheffrahn 2000; Evans et al. 2013), favouring termite populations in places where their presence was previously limited by these factors (Lal 2004; Peterson 2010; Lee & Chon 2011; Guerreiro et al. 2014). There is thus a need to develop efficient preventive and control methods for avoiding possible future termite pests' outbreaks.

Subterranean termite control strategies are studied and applied worldwide and mainly rely on the use of chemical or physical barriers, wood treatment with insecticides or wood modification by acetylation, furfurylation or other techniques, and subterranean termite population control using baits. Few of the primary issues in termite control are the need to efficiently kill the entire colony and the durable protection of the materials. Difficulties arise due to the cryptic and diffuse nature of rhinotermitid pest termite species, which forage either in extensive underground galleries, build nests hidden underneath the soil surface, or live in small colonies inside the wood they infest. Subterranean termite control has relied on the use of persistent, broad spectrum insecticides applied to the soil beneath structures. Therefore, in the last few decades, although much remains to be investigated, integrated pest management strategies (IPM) have been favoured, such as local spot treatment of infested timber and population control through the use of baits and insect growth regulators (like chitin synthesis inhibitors) (Evans & Iqbal 2014).

If the biology and ecology of the pest species are considered, management strategies can potentially become more specific, and therefore also potentially more sustainable. Further understanding of how termites feed and obtain nutrients and grow as a colony will assist in developing greatly needed innovative termite control methods.

LIGNOCELLULOSE DIGESTION

FEEDING SUBSTRATE

Wood is a natural material composed of three main types of components: cellulose (framework substance), hemicellulose (matrix substance present between cellulose microfibrils) and lignin (incrusting substance for cell wall solidification).

For the digestion of these main components, several enzymes are needed; some of those enzymes are not yet identified in lower termites, such as exoglucanases or hemicellulases, and enzymes present inside the flagellate protists, enabling the provision of these cellulases during lignocellulose digestion (Hongoh 2011). Cellulases are enzymes which have the ability to produce sugars from crystalline cellulose (Slaytor 1992). Cellulose and hemicellulose are thus degraded to sugars, which are then processed into acetate, hydrogen and carbon dioxide. These products may be used directly by the termite or may interact with other nutrients processed inside termite hindgut. Acetate is used as the main energy source of the termite (Breznak 1982).

DIGESTIVE TRACT

Termites' ability to efficiently digest cellulose relies not only on chemical features (cellulolytic enzymes), but also on the digestive tract's physiological properties. The termite digestive tract is composed of different parts: mouth, salivary glands, foregut, midgut and hindgut (Fig. 2; salivary glands not shown in this figure), and each part has a specific function in terms of lignocellulose breakdown. The lignocellulose breakdown starts in the mouth, with the use of solid and hardened mandibles to chew the wooden substrates; the crop and the proventriculus are additional organs situated in the foregut, which are responsible for further milling and filtering of the ingested wood particles (Watanabe & Tokuda 2010; Brune & Ohkuma 2011). Indeed this physical conditioning of the food is crucial for efficient digestion as it results in proper cleavage of the substrate and thus facilitates the access of cellulolytic enzymes.

The hindgut harbours a rich symbiotic fauna, which was thought to be parasitic in the early years of research. Only after Cleveland (1923) was clear that termites devoid of hindgut symbionts

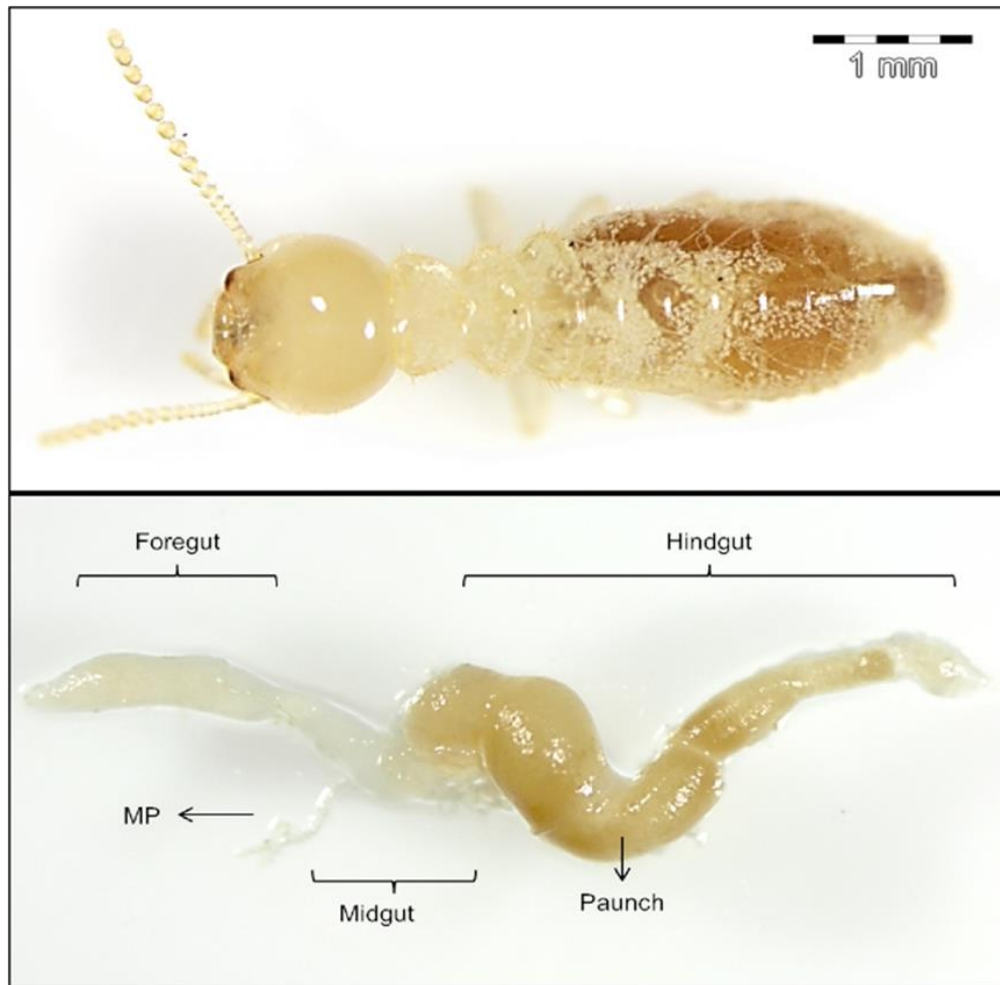


Fig. 2. Photo showing a worker and the extracted gut with different parts of the subterranean termite *Reticulitermes grassei* Clément gut: Foregut; Midgut, including the Malpighian tubules (MP) at the posterior end of the midgut; Hindgut

were unable to digest lignocelluloses fully, suggesting that the relation between host termite and the hindgut fauna was nutritional symbiosis. Since then, the evidence of flagellate protists' contribution to lignocellulose digestion, through their cellulolytic enzymes, has been demonstrated and is widely accepted (e.g. Yamin & Trager 1979;

Yamin 1980; Slaytor 1992; Yoshimura et al. 1996; Inoue et al. 1997; Scharf et al. 2011a; Xie et al. 2012; Tsukagoshi et al. 2014).

The enlarged hindgut is a key structure for lower termites' ability to digest lignocellulose efficiently, as it concentrates major chemical action on cellulose, with a dilated paunch

harbouring the majority of symbiotic fauna. Though fairly simple in lower termites, the anterior and posterior paunch, and the anterior and posterior colon are sequentially structured for digestion, each serving as defined micro-niches in terms of gradients of oxygen, hydrogen and pH, created by a combination of host and hindgut symbiont activities (Brune & Friedrich 2000), creating difficulties for researchers to reproduce the physical-chemical conditions of the hindgut within the laboratory. Radial concentrations of oxygen and hydrogen showed a peripheral zone of the hindgut where oxygen is available, and enabling the survival of both aerobic or facultative aerobic microorganisms, whereas in the hindgut centre, an anaerobic environment is established, with a zone with high hydrogen concentration, resulting from the activity of flagellate protists, which release hydrogen, carbon dioxide and acetate and are anaerobic (Brune et al. 1995). The hydrogen and carbon dioxide produced is then used by prokaryotes for methanogenesis or acetogenesis, whereas acetate may be used by the termite host (Brune 1998, 2013). Furthermore, to harbour anaerobic flagellate protists, the termite hindgut functions as an oxygen sink and this gas needs to be consumed (Brune et al. 1995). Therefore, flagellate protists are often associated with methanogenic bacteria and other facultative or strict aerobic hindgut microbiota which consume the oxygen, maintaining an anoxic environment in some parts of the paunch (Brune 1998; Brune & Friedrich 2000).

Additionally, endogenous cellulases in lower termites have been identified and are harboured mainly in the salivary glands, playing an important role in the lignocellulose degradation process (Slaytor 1992; Watanabe et al. 1998; Scharf & Tartar 2008; Scharf et al. 2011a; König et al. 2013; Peterson et al. 2015). The lignocellulose digestion is probably the result of synergistic action of both termites and their symbionts; though the degree of nutritional mutualism has some times been questioned (Scharf et al. 2011a; Scharf 2015). Recent metatranscriptomic analysis of termite hindgut content has indeed confirmed that the lignocellulolytic system has a tripartite origin: protists, bacteria and termites (Xie et al. 2012; Peterson et al. 2015).

MULTI-LEVEL SYMBIOSIS

TERMITE AND ITS SYMBIOTIC FAUNA AS A HOLOBI- ONT

Symbiosis was defined by Anton de Bary in 1879 as the ‘living together of different species’; however, this definition does not describe the complex nature of the relation between termites and their gut symbiotic fauna. The highly complex ecosystem with different levels of symbiosis inside termite guts is the result of complex and diverse evolutionary events and also of the singular social behaviour of termites. This relationship certainly goes beyond the Anton de Bary concept of symbiosis. Besides the hindgut symbiotic microbiota, the termite colony has also been considered as an organism, since the basic functions are clearly divided in its different parts: reproduction and dispersion (queens, kings and alates), construction, feeding and tending (workers), active defence (soldiers) and protection, homeostasis and fortification (nest) (Eggleton 2011). The gut symbiotic fauna may be directly involved not only in feeding functions, but also in tending, defence, and homeostasis (Matsuura 2001; Ugelvig & Cremer 2012; Chouvenc et al. 2013; Sen et al. 2015). The symbiotic fauna is probably also involved in shaping the termite social behaviour. For example, recently it was shown that lower termites exhibit different undertaking behaviour towards conspecific (necrophagy) or congeneric (burial behaviour) termite corpses. This behaviour was interpreted as a defence mechanism together with a cost mitigation strategy (Sun et al. 2013). Another possible advantage of this behaviour may be the protection of the hindgut symbiotic fauna, avoiding the introduction of new elements into the hindgut fauna by the ingestion of corpses of termites belonging to other species. The term holobiont has been accepted to refer to the host and its microbiota as a whole unit able to live, develop, survive and evolve together (Rosenberg & Zilber-Rosenberg 2013; Scharf 2015). The cooperation between hosts and their microbiota results in a positive contribution to the fitness of the association, providing an increased ability to adapt more rapidly to changing conditions (Rosenberg & Zilber-Rosenberg 2013). The capacity to tackle imposed changes and stresses results from the

synergy of combined capacities. Furthermore, social insects such as termites are robust in resisting genetic diversity losses during phenomena such as introductions or other sources of low population genetic bottlenecks (Ugelvig & Cremer 2012). From this perspective, it is clear that fitness changes in the termite colony can be induced at different levels.

Unarguably, the symbiotic association between lower termites and their hindgut symbionts has advantages for both, since the termites are able to receive contributions to their main energy supply resulting from lignocellulose digestion, and hindgut symbionts have shelter, protection and food, supplied by the termite host (Radek 1999; Noda et al. 2009; Brune 2013; Tamschick & Radek 2013). Probably, the nature of the symbiotic fauna, and the relationships between and amongst them, are driven by host social behaviours and shifts in feeding habits. However, some have argued that the symbiotic microbes lead to the development of social habits in wood-feeding insects (termites and cockroaches) (e.g. Nalepa et al. 2001), so the paradox remains. Nevertheless, symbionts may impose important selective pressures on their hosts, as well as diet preferences or feeding habits (Rosengaus et al. 2011; Xiang et al. 2012; King et al. 2013).

The tripartite symbiosis between termite host, flagellate protists, and prokaryotes responsible for lignocellulose degradation translates into a unique symbiotic community, which is probably under strong co-evolutionary pressure. Indeed, the majority of gut microbiota are considered to be autochthonous symbionts likely to have co-evolved with their termite host species (Hongoh et al. 2005; Noda et al. 2007; Tai et al. 2015). The invasion of new habitats or the proximity of different termite species does not seem to influence the gut symbiotic fauna structure of termite species, which tends to maintain its integrity in terms of diversity of symbiont species (Kitade 2004; Hongoh et al. 2005; Husseneder et al. 2010; Boucias et al. 2013). Within a termite colony, a rather species-specific gut symbiotic fauna is expected, as termites rely on horizontal transmission of hindgut symbionts to recover the hindgut symbiotic community, since, when they moult, symbionts are also discarded. The recovery is done by proctodeal or stomodeal trophallaxis.

This within-nest symbiont transmission was observed in a genomic and metagenomic study of a fungus-growing termite (Poulsen et al. 2014). The obligatory vertical mode of transmission of the gut symbiotic fauna to the next generation probably determines the gut symbiotic structure associated with termite species colonies, leading thus to higher levels of host-symbiont specificity (Hongoh et al. 2005; Noda et al. 2007; Husseneder 2010).

Some evidences of co-evolution between flagellate protists and their host termites rely on phylogenetic analyses which show a clear co-diversification pattern, although factors such as stochastic, dietary and ecological effects are also important in the long term evolution of symbiont communities (Tai et al. 2015). Parabasalid protists seem to be strongly influenced by host phylogeny, and the symbiotic bacteria communities seem to be more influenced by dispersal and environmental acquisition (Tai et al. 2015). Within the parabasalids, the Cristamonadea class seems to be strongly associated with the Kalotermitidae family, whereas the Spirotrichonymphea class is linked with the Rhinotermitidae termite family (Tai et al. 2015).

In contrast, flagellate protists belonging to the genus *Trichonympha* are widely distributed and were detected in six different termite families and also inside the wood-feeding cockroaches belonging to the genus *Cryptocercus*. This supports the hypothesis that *Trichonympha* spp. symbionts were acquired by the most recent common ancestor of termites and wood-feeding *Cryptocercus* spp. cockroaches (Inward et al. 2007a; Carpenter et al. 2009; Ikeda-Ohtsubo & Brune 2009; Tai et al. 2015). It is important to highlight, however, that some authors found a greater cryptic diversity of *Trichonympha* species inside one host, using molecular analyses, than the diversity predicted by use of morphological analyses only (James et al. 2012; Tai et al. 2013). This suggests that termite hindgut diversity estimations could be biased because of the underestimation of symbiont diversity, especially considering that *Trichonympha* spp. are among the largest species of flagellate protists living inside termites (James et al. 2012; Tai et al. 2013).

Intracellular bacterial symbionts of termite hindgut flagellates belonging to Endomicrobia (phylum

Termite Group 1) tend to form a unique phylogenetic lineage with either their flagellate protist host species or their termite host, suggesting co-speciation events (Ikeda-Ohtsubo et al. 2007; Tai et al. 2015). Co-speciation between *Bacteroidales* endosymbionts and their host protists was demonstrated in 13 out of 14 taxa of the protist genus *Pseudotriconympha*, and in turn these protist species showed an almost complete co-speciation with host termite species belonging to the Rhinotermitidae (Noda et al. 2007). In a recent study of the 'Endomicrobia' associated with *Triconympha* genus, strict host specificity was concluded (Zheng et al. 2015). In contrast, oxymonads' and parabasalids' 'Endomicrobia' symbionts are rather similar between flagellate protist hosts, suggesting that they are horizontally transmitted among different flagellate protist species living inside the same termite hindgut, accounting for the high levels of symbiont transfer between flagellate protist hosts and thus lacking (or having reduced levels) of host-symbiont specificity (Ikeda-Ohtsubo et al. 2007; Ikeda-Ohtsubo & Brune 2009).

An obligatory ectosymbiont of the flagellate protist *Devescovina* spp. has been identified, suggesting that this highly specific relationship evolved as a consequence of strong metabolic interactions (Desai et al. 2010).

At another level of this symbiotic network, the adaptation of morphological characters of both host protists and ecto- and endosymbiotic bacteria has been observed (Noda et al. 2007, 2009). Specialised cell-surface features which facilitate bacterial attachment indicate a close integration of these ectosymbionts in the protist metabolism, and corroborate the co-evolution hypothesis on their symbiotic relationships (Radek et al. 1992; Dolan et al. 2000).

HINDGUT PROKARYOTES

Most of the hindgut prokaryotes are bacteria, whereas the Archaea represent a low percentage of hindgut symbionts. The major groups of bacteria usually identified in lower termite hindgut are: *Spirochaetes*, *Bacteroidetes*, *Elusimicrobia* and *Firmicutes* (Clostridiales) (Stingl et al. 2005; Boucias et al. 2013; Brune 2013). The termite hindgut has proven to be a rich source of novel

organisms, including new findings for science at high taxonomic levels (Brune 1998; Ohkuma 2003; Boucias et al. 2013; Sato et al. 2014; Tai et al. 2015).

The maintenance of an anoxic environment in some parts of the paunch is thought to be just one of the many putative roles of the symbiotic bacteria. These bacteria exhibit different levels of association: free-living in the hindgut (symbiotic with the insect host), directly associated with flagellate protists (either as endo- or as ectosymbionts) or associated with the gut wall (Tamschick & Radek 2013). The tasks of symbiotic bacteria (prokaryotes) and their roles are not completely understood and many species remain undescribed (for a review on this issue see Brune 2013). Brune (2013) defined different types of hindgut prokaryotes including: lignocellulolytic bacteria, bacteria involved in oxygen reduction reactions, fermentation bacteria, bacteria responsible for hydrogen metabolism and nitrogen-fixing bacteria.

Recently, cultivation-independent studies helped to identify and catalogue hindgut prokaryotes in relation to their function inside the termite hindgut, including some evidences on their role in lignocellulose digestion (Warnecke et al. 2007; Boucias et al. 2013; He et al. 2013; King et al. 2013; Peterson et al. 2015). Advances in sequencing and its accessibility raise expectations regarding future unveiling of the role of bacteria inside the termite hindgut.

FLAGELLATE PROTISTS

Flagellate protists are part of the unicellular eukaryotes belonging to two separate lineages: the order Oxymonadida (Phylum Preaxostyla) and the Phylum Parabasalia (Brugerolle 1991; Moriya et al. 1998; Čepička et al. 2010; Adl et al. 2012). In spite of the difficulties in laboratory cultivation of most of these organisms, their taxonomy was initially based on morphological characters (e.g. Brugerolle 1991; Cavalier-Smith 1993). With culture-independent techniques it has become possible to reconstruct the phylogeny of some groups of flagellate protists, and some studies are based on both morphological and molecular data (e.g. Carpenter et al. 2010; Čepička et al. 2010).

Table 1. Key diagnostic characters of the major groups of symbiotic flagellate protists living inside lower termites' intestine belonging to the phyla Parabasalida and Preaxostyla (order Oxymonadida) (adapted from: Brugerolle & Lee 2000; Čepicka et al. 2010; Adl et al. 2012; Radek et al. 2014).

	Flagellar apparatus/Mastigont system				Cytoskeletal arrangement		
	Closed mitosis	Golgi body	Hydrogenosomes	Nuclei	Flagellar system	Basal bodies arrangement	Microtubular fibers/sheets
Parabasalia							
Class Hypotrichomonadea	+	+	+	+	Basically: 4 flagella	Basically: 4 basal bodies, 3 anterior, 1 recurrent	Pelta - covers the anterior part of the cell
Class Trichomonadea	+	+	+	+	4	biramous parabasal body	
Class Tritrichomonadea	+	+	+	+	2 - 6	single karyomastigont	
Class Cristamonadea	+	+	+	1 or 2	0 - 5	single karyomastigont	pelta-axostyle complex may be reduced or absent
Class Trichonymphea	+	+	+	1 or more	2 to thousands per mastigont	single to multiple mastigonts	often spiralised or ramified axostyle
Class Spirotrichonymphea	+	+	+	1	hundreds to thousands	cell body divided into anterior rostrum and post-rostral area	thin and numerous, do not protrude outside the cell
Preaxostyla							
Order Oxymonadida	+	+	+	1	4, or a multiple of 4, flagella arranged in pairs	kinetosomes arranged into spiraled rows	tube-like and stout; multiple thin bands, or reduced
	internal spindle			1 or more		Preaxostylar lamina linking the 2 basal body pairs; Pelta (caps the nucleus)	crystalline axostyle; microtubular rod extending through the entire length of the cell

The oxymonads do not have energy-generating organelles or a highly developed intracellular membrane systems, like dictyosomes, and live mostly inside insects. Oxymonads may be motile or attached to termite hindgut walls. The most common oxymonads living inside termite hindgut belong to the genera *Dinenympha*, *Pyronympha* or *Oxymonas* (Brugerolle & Radek 2006). In contrast to the oxymonads, the parabasalids have anaerobic energy-producing organelles (hydrogenosomes) and a characteristic parabasal apparatus, which is comprised of dictyosomes associated with parabasal fibres. Parabasalids may be large and are highly motile, with four or more flagella. These protists live mostly inside termites and cockroaches as symbionts, but some species may be parasites or commensals of vertebrate hosts (Čepička et al. 2010). Table 1 summarises information on flagellate key diagnostic characters (additional information on the flagellate protists' internal structures is also available in Table 1 of the supplementary material). Flagellate protists are strictly anaerobic and ferment cellulose to acetate (Yamin 1980; Yoshimura et al. 1996; Hongoh 2011). Acetate is also important as a precursor for the synthesis of other products as amino acids or cuticular hydrocarbons (Breznak 1982). Carbon dioxide and hydrogen, two other products of cellulose fermentation, are used by the anaerobic bacteria mainly to produce acetate through reductive acetogenesis (Hongoh 2011).

Protists represent the majority of termite hindgut microorganisms (Katzin & Kirby 1939; Inoue et al. 1997; König et al. 2013). The general uniqueness of these protists to termites and to wood-feeding cockroaches belonging to the genus *Cryptocercus* highlights the origin of termites from a cockroach-like ancestor, corroborated by molecular phylogenetic data (Inward et al. 2007a). The mode of transmission of protists and the close relationships developed between host and symbionts in terms of metabolic interactions and needs probably account for the developed specificity (Kitade et al. 2012). Based on recent attempts to infer termite family relationships (Engel et al. 2009; Bourguignon et al. 2015), and according to available data on flagellate protist classes (Parabasalia) and order (Preaxostyla) identified to date, a gradual tendency towards diversification of the symbiotic community inside the termite hosts is evident. However, the symbiotic community has been researched only in a small number of termite species and rather asymmetrically, with a preponderance in some families. Plotting the number of flagellate protist groups per termite host family, it is possible to see an increase in diversity along host evolutionary pathway until the point at which termites completely lose their flagellate protists and are able to switch to a more diversified diet (family Termitidae) (Fig. 3).

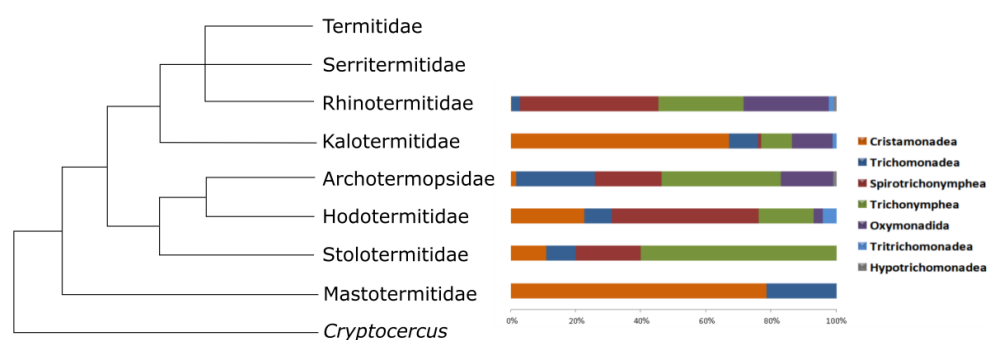


Fig. 3. Simplified scheme of phylogenetic relationships within termites (based on Engel et al. 2009 and Bourguignon et al. 2015; see these authors for further details) and the number of flagellate protist species belonging to classes (Parabasalia) and order (Preaxostyla) identified to date inside termites.

The Rhinotermitidae family is not an exception, and a similar pattern is observed when the subfamilies are analysed in terms of flagellate protist diversity: the basal subfamilies (Rhinotermitinae, Prorhinotermitinae, Psammotermitinae, Termitogetoninae) have three or fewer flagellate protist groups, whereas Coptotermitinae and Heterotermitinae, considered to be the sister groups of the family Termitidae (Bourguignon et al. 2015), exhibit a higher taxonomic diversity (four to six) of flagellate protists groups.

Different types of events may have occurred during termite diversification, e.g., the external uptake of symbionts or the horizontal transmission of symbionts within different termite species which were foraging the same area and resources. The diversification of termites towards an optimisation of the digestive process, depending on the environmental conditions and type of resources exploited by the termites, are important factors to explain the flagellate protist communities associated with certain groups of termites. Further insights into the evolutionary pathways and constraints driving the relationships among termites and their symbiotic flagellate protist communities are needed. This may shed light on the mechanisms and driving forces which determine the establishment of symbiotic relationships among different organisms and give rise to holobiotic forms of life.

Many flagellate protists are considered species-specific, such as *Pyrsonympha vertens* Leidy and *Joenia annectens* Grassi that have only been identified inside the hindgut of *Reticulitermes flavipes* (Kollar) and *Kaloterms flavicollis* (Fabricius), respectively. Whereas *Holomastigotes elongatum* (Grassi) seems to be a species with less host-specificity, as it was identified inside 12 different termite species belonging to three different families (Archotermopsidae, Hodotermitidae and Rhinotermitidae). However, this finding may be questionable as these protists' identification relied only on morphological characters evaluated by different authors (Harper et al. 2009). In some termite species, such as *Coptotermes heimi* (Wasmann), *Heterotermes indicola* (Wasmann), *Cryptotermes havilandi* (Sjöstedt) or *R. flavipes* more than 20 different flagellate protists have been identified to date (*C.*

heimi, *H. indicola* and *C. havilandi*: Yamin 1979 and references therein, Desai et al. 2010; *R. flavipes*: Leidy 1877; Mello 1920; Cleveland 1923; Breznak & Pankratz 1977; Mauldin et al. 1977; Bloodgood et al. 1975; Mauldin et al. 1981; Lelis 1992; Cook & Gold 1998; Stingl & Brune 2003, Stingl et al. 2005; Brugerolle 2006, Brugerolle & Bordereau 2006, Lewis & Forschler 2006; Hu 2008; Lewis & Forschler 2010; Hu et al. 2011, Tamschick & Radek 2013; and references therein: Kudo 1939; Ghidini 1942; Yamin 1979; Grassé 1982). However, other termite species may have a less diverse flagellate protist community, such as *Incisitermes snyderi* (Light), with only three identified species of flagellate protists (Dolan et al. 2000; Gerbod et al. 2002; Harper et al. 2009). As an example of a diverse flagellate protist community, inside the European subterranean termite from Portugal, *Reticulitermes grassei* Clément, 12 different morphotypes were identified based on morphological traits. The European subterranean termite *R. grassei* (Rhinotermitidae) is native of the Iberian Peninsula, and also present in the Atlantic coast of France (Kutnik et al. 2004). One invasive population of *R. grassei* was identified in the UK (Jenkins et al. 2001), and another in Faial Island of the Azores (Ferreira et al. 2013). The flagellate community dynamics after an invasive event may be of major interest for understanding the mechanisms of adaptation to new environments of subterranean termites. The flagellate protist community from *R. grassei* is presumed to be dominated by *Pyrsonympha* sp. (42.4%), *Microjoenia hexamitoides* Grassi (13.4%), *Dinenympha gracilis* Leidy (10.4%), and *Spirotrichonympha flagellata* Grassi (5.4%); *Trichonympha agilis* Leidy (4.3%), *Hexamastix* sp. (4.0%) and *Holomastigotes elongatum* Grassi (3.3%) were also represented in all termites observed (Duarte et al. 2016).

Flagellate protist identification errors owed to over- or underestimation of the flagellate protist community living inside a termite hindgut are common. The misidentification of the different life cycle stages of one flagellate protist species as a different species is an example of overestimation of flagellate protist diversity. Underestimation errors may also occur because of: 1) manipulation constraints; 2) lack of identification power of the DNA markers used; 3) the frequent concentration

of research efforts on the larger species, which are more easily analysed morphologically, overlooking the smaller ones, and 4) misidentification of different similar species as an only species through morphological analysis (Harper et al. 2009; James et al. 2013; Tai et al. 2013). Nonetheless, recent advances in genomic and metagenomic techniques are leading to higher quality and affordable community sequence strategies. As a consequence, metagenomic data on gut community of different termite host species are increasing. However, the lack of correspondence of operational taxonomic units obtained with metagenomic analyses with known flagellate protist species is a drawback that requires further taxonomic research efforts. A resilient bottleneck still remains regarding suitable genetic markers that are taxonomically and/or functionally valid and have enough discrimination power regarding downstream data analyses.

The diversity of flagellate protist fauna associated with lower termites could be explained by a strong division of the labour required to accomplish the intricate process of lignocellulose digestion; a species or group of species acts in specific phases of this process (Yoshimura et al. 1996; Inoue et al. 1997; Todaka et al. 2007; Raychoudhury et al. 2013).

POTENTIAL SPIN-OFFS

BIOTECHNOLOGY APPLICATIONS

Lignocellulose is one of the most abundant renewable types of biomass available on earth, and cellulose-based biofuels are considered to be a sustainable alternative to reduce our dependence on fossil fuels (Ragauskas et al. 2006; Yang & Wyman 2008). However, the industrial processing of lignocellulose conversion to energy needs to be adjusted in order to reduce costs and improve energy production and consequently compete with fossil fuels. For example, current biological conversion of cellulosic biomass for bioethanol production is based on bacterial and fungal cellulolytic systems (Sun & Scharf 2010). The most expensive part of bioethanol production is the pretreatment (Ragauskas et al. 2006; Yang & Wyman 2008). The information on the identification and characterisation of endogenous and symbiotic genes and enzymes from an effective natural bioreactor, such as

subterranean termite gut, is relevant for the improvement of industrial processes (Helle et al. 2004; Brune 2007; Yang & Wyman 2008; Scharf & Boucias 2010; Tsukagoshi et al. 2014). Cellulolytic enzymes and genes from termites and their symbiotic fauna are potential candidates for integrating, and consequently refining, bioethanol production technologies, by the identification of relevant catalysts and/or by the discovery of potential recombinant enzymes which enable the maximum efficiency of the processes; it is also possible to use mutagenesis in order to functionally improve enzymes (e.g. Helle et al. 2004; Scharf & Tartar 2008; Husseneder 2010; Scharf & Boucias 2010; Zhang et al. 2010; König et al. 2013).

Other technical processes could benefit from deep knowledge of termite and symbiont interactions inside termite gut. For example, the breakdown of lignocellulose into shorter structural elements can form the chemical building blocks for the production of new synthetic materials (Ragauskas et al. 2006). The intestinal tract of termites is a rich source of glycanolytic microorganisms, which may be used for other applications, such as the prevention of slime production and other undesirable side-products during vinification (Blättel et al. 2011) or for bioremediation purposes, because of their ability to degrade toxic substances (Ke et al. 2012). Furthermore, it may also be possible to use termite symbionts for nitrogen fixation in soil fertilisation (Husseneder 2010; Du et al. 2012; Thong-On et al. 2012).

NEXT-GENERATION TERMITICIDES

Termite gut microbiota and respective cellulosic activity may be a strategic target for designing molecular-based bio-pesticides for termite control (Zhang et al. 2010). The effectiveness of the potential biological control agents previously studied has been compromised because of the symbiotic hindgut fauna, which has a protective role regarding novel and potentially harmful microorganisms, of the termite immune system and hygienic behaviour, such as grooming activities and burying and isolation of dead termites (Chouvenc & Su 2012; Sun et al. 2013). A recent study on the synergistic effects of using a nicotinoid and a pathogenic agent showed the potential of this mixture to disrupt termite social behaviour and cause deleterious effects on the colonies. One of the major effects of this

treatment was the decrease of the flagellate protist populations living inside the termites (Sen et al. 2015). Knowledge on lignocellulose digestion processes may allow the definition of potential targets for novel termite control strategies based on an alternative mode of action approach. The development of next-generation termiticides, targeting cellulolytic activities encoding genes, of endogenous or symbiont origin, with RNA interference techniques has proven to be possible (Zhou et al. 2008; Itakura et al. 2009; Scharf et al. 2011b; for a review of RNA interference advances in termites and/or symbiotic protists see Scharf 2015). For example, a high-dose stranded RNA force feeding trial led to the silencing of two termite genes, one of them involved in cellulose digestion, the other in caste differentiation, and this led to an increase in mortality in the experimental population (Zhou et al. 2008).

Paratransgenesis represents a target-specific strategy, which relies on the manipulation of genetically engineered natural symbionts (gut bacteria) which will act as a Trojan horse, as they are capable of surviving inside termite gut while carrying and expressing toxins which are then spread throughout the colony by social interactions (Douglas 2007; Husseneder et al. 2010; Rangberg et al. 2012, Sethi et al. 2014). The conjugation of this technique with a ligand-lytic peptide, which will enable the design of specific ligands for flagellate protists, has already proven to be effective against lower termite pests (Husseneder et al. 2010; Sethi et al. 2014). Further applications of this technology may involve the control of other insect pests which harbour flagellate protists and/or may act as vectors of protists; also, the technique could be refined to develop drugs against disease-causing protists (Sethi et al. 2014).

IMMUNITY AGENTS ROLE AND OTHER APPLICATIONS

Termite gut symbiotic microbiota is an active part of the efficient immunity system of termites (Chouvenc et al. 2013). Studies on free-living heterotrophic protists in water discovered their important role in eliminating viruses by feeding on them (Deng et al. 2014). Some flagellate protists inside termites may prey on bacteria by ingestion (e.g. Noda et al. 2009); this process may also imply eventual feeding on viruses. Possibly some protists

assume the same role inside termites, and even inside other animals, acting as potential elimination agents of viruses or other pathogenic agents.

OVERVIEW

Our attitude towards the described organism, whether for searching for more effective control strategies or for determining its correct use as a biotechnology model, should shift from the individual termite and its gut microbiota as separate entities towards a more holistic approach considering this holobiont as an independent, evolutionary and functional unit. By adding an ecological and environmental axis to this holobiotic approach, we will be better able to integrate protists' diversity and ecology, contributing to further applications such as: 1) understanding the co-evolution mechanisms that lead to the establishment of this highly efficient natural bioreactor and its consequent ability to convert lignocellulose into energy sources (Tai et al. 2015); 2) the possible adjustment of diverse technical industrial processes such as a biorefinery (Scharf & Tartar 2008; Scharf 2015); and 3) the application of novel strategies for a more sustainable termite control in urban environments (Husseneder 2010; Scharf 2015).

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ANNEX 1 – SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1.

Description of the internal structures and body of the flagellate protists living inside lower termites and belonging to the phyla Parabasalia and Preaxostyla (order Oxymonadida). Adapted from: Honigberg et al.(1971); Brugerolle & Lee(2000); Brugerolle & Radek(2006); Čepička et al. (2010); Radek et al. (2014).

Structure	Description/Location
Centrioles	pair of organelles that form the core of the centrosome located near to the nucleus
Centrosome	structure which function as a microtubulus organizing centre located near to the nucleus
Basal bodies or kinetosomes	modified centrioles that give rise to cilia and flagella below the cell membrane
Mastigont or kinetid	kinetosome(s) with its cytoskeletal root system below the cell membrane
Karyomastigont	mastigont plus its own associated nucleus
Axostyle	microtubular structure which forms the cell axis, made of sheets of microtubules spiralized, or lying in parallel layers (oxymonads), to a hollow or filled tube or cone
Pelta	second microtubular sheet covering the anterior end of the cell
Recurrent flagellum (RF)	backwardly directed flagellum, running posteriorly over the body of the flagellate in a loose or attached state; when attached it often becomes part of an undulating membrane
Undulating membrane	RF adherent to a projection of the cytoplasmatic membrane (typical arrangement), or the projection may also be the flagellum itself adjacent to the cytoplasmatic membrane
Costa	non-microtubular striated fiber, type A or B according with the pattern of striation underlain the undulating membrane specific for Parabasalia
Cresta	fibrillar, noncontractile structure, with subtriangular shape located below the basal portion of the trailing flagellum characteristic of devescovid flagellates
Parabasal body	modified Golgi dictyosome characteristic of the Parabasalia
Parabasal apparatus	complex consisting of a parabasal bodies attached to striated fibers
Rostrum	the apical end or tip of a protozoan body, when its shape is that of a beak is some other sort of distinctive protuberance in that area of the body anterior part of the cell
Flagellar apparatus	basal bodies of the flagella and their connected roots
Holdfast	any structure by which a given organism can attach, temporary or permanently, to some living or inanimate substrate
Infrakinetosomal body (IFK)	large, dense structure below the basal body complex
Suprakinetosomal body	crescent-shaped structure anterior to, and connecting, with kinetosome no.2 associated with IFK
Comb-like structure	periodic structure, comb-shaped extends between costa and IFK